

Mechanisms Involved in the Selection of HIV-1 Reverse Transcriptase Thumb Subdomain Polymorphisms Associated with Nucleoside Analogue Therapy Failure^{▽†}

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Previous studies showed an increased prevalence of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) thumb subdomain polymorphisms Pro272, Arg277, and Thr286 in patients failing therapy with nucleoside analogue combinations. Interestingly, wild-type HIV-1_{BH10} RT contains Pro272, Arg277, and Thr286. Here, we demonstrate that in the presence of zidovudine, HIV-1_{BH10} RT mutations P272A/R277K/T286A produce a significant reduction of the viral replication capacity in peripheral blood mononuclear cells in both the absence and presence of M41L/T215Y. In studies carried out with recombinant enzymes, we show that RT thumb subdomain mutations decrease primer-unblocking activity on RNA/DNA complexes, but not on DNA/DNA template-primers. These effects were observed with primers terminated with thymidine analogues (i.e., zidovudine and stavudine) and carbovir (the relevant derivative of abacavir) and were more pronounced when mutations were introduced in the wild-type HIV-1_{BH10} RT sequence context. RT thumb subdomain mutations increased by 2-fold the apparent dissociation equilibrium constant (K_d) for RNA/DNA without affecting the K_d for DNA/DNA substrates. RNase H assays carried out with RNA/DNA complexes did not reveal an increase in the reaction rate or in secondary cleavage events that could account for the decreased excision activity. The interaction of Arg277 with the phosphate backbone of the RNA template in HIV-1 RT bound to RNA/DNA and the location of Thr286 close to the RNA strand are consistent with thumb polymorphisms playing a role in decreasing nucleoside RT inhibitor excision activity on RNA/DNA template-primers by affecting interactions with the template-primer duplex without involvement of the RNase H activity of the enzyme.

Human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) is a major target for antiretroviral drug development (3, 61). HIV-1 RT catalyzes the conversion of the viral single-stranded genomic RNA into a double-stranded DNA that integrates into the host genome. HIV-1 RT is a heterodimer composed of 66- and 51-kDa subunits, with subdomains termed fingers, thumb, palm, and connection in both subunits and an RNase H domain in the large subunit only (23, 26, 29).

Approved antiretroviral drugs targeting the HIV-1 RT include nucleoside and nucleotide inhibitors (zidovudine, lamivudine, stavudine, didanosine, zalcitabine, abacavir, emtricitabine, and tenofovir) and nonnucleoside inhibitors (nevirapine, delavirdine, efavirenz, and etravirine) (39). Nucleoside RT inhibitors (NRTIs) mimic natural nucleosides. Inside the cell, nucleoside (and nucleotide) analogues need

to be phosphorylated to their active triphosphate forms to act as competitive inhibitors of HIV-1 RT. Since NRTIs lack a 3'-OH group, their incorporation results in chain termination (12, 38, 62). Despite the efficacy of current antiretroviral therapies, the selection and emergence of drug-resistant HIV-1 strains are major factors contributing to treatment failure.

HIV-1 RT mutations conferring resistance to nucleoside (or nucleotide) inhibitors act either by (i) improving discrimination against the RT inhibitors (15, 54, 59) or (ii) by increasing the RT's ability to remove 3'-terminal chain terminator inhibitors from blocked DNA primers, through phosphorolysis mediated by ATP or pyrophosphate (PP_i) (2, 41). The nucleotide excision or primer-unblocking mechanism appears to be most relevant for the acquisition of resistance to thymidine analogues, such as zidovudine (AZT) (3'-azido-3'-deoxythymidine) and stavudine (d4T) (2',3'-didehydro-2',3'-dideoxythymidine), through the accumulation of the so-called thymidine analogue resistance mutations (TAMs) (i.e., M41L, D67N, K70R, L210W, T215F or T215Y, and K219E or K219Q) (for recent reviews, see references 38 and 68). TAMs increase the rate of nucleotide excision. Although primer-unblocking efficiencies are influenced by the nucleotide sequence context (44), available data indicate that thymidine analogues and

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tenofovir are the best substrates of the reaction, while cytidine analogues are removed very inefficiently (7, 25, 31, 33, 41, 42, 46, 55, 63, 72). Despite being excisable, there is no agreement on the unblocking efficiencies for primers terminated with carboxy monophosphate (CBVMP) (46, 56, 72) or dideoxyadenosine monophosphate (ddAMP) (25, 43, 46). CBVMP and ddAMP are active metabolites of abacavir and didanosine, respectively.

Computational analysis of the HIV genetic variability in the RT-coding region revealed an association between the presence of TAMs and mutations at codons 35, 39, 43, 122, 203, 207, 208, 214, 218, 223, and 228 in patients failing therapy with RT inhibitors (9, 11, 65). Some of those mutations appear to increase viral fitness in the presence of TAMs, as demonstrated for K43E (24), Q207D (30), and L214F (52). However, very often, genotypic analysis is restricted to RT residues 1 to 240, while growing evidence suggests that potential antiretroviral therapy-related mutations in the thumb-connection subdomains (codons 241 to 424) and RNase H domains (codons 425 to 560) can likewise contribute to resistance to RT inhibitors. Thus, recent reports have suggested that mutations in the connection subdomain (e.g., E312Q, G335C/D, N348I, A360I/V, V365I, and A376S) and in the RNase H domain of the RT (e.g., Q509L, H539N, or D549N) can significantly increase zidovudine resistance by altering the balance between excision and template RNA degradation (13, 47, 48, 73). A reduction of the specific RNase H activity of the viral RT stabilizes the RNA/DNA duplex, giving RT more time to excise the AZT from the terminated primer (8, 14, 18).

The analysis of HIV-1 subtype B genomes of isolates obtained from untreated patients reveals three major polymorphisms within the RT thumb subdomain at positions 272, 277, and 286 (Stanford University HIV Drug Resistance Database [<http://hivdb.stanford.edu>]). Ala and Pro were found at position 272 in 47.4% and 44.0% of the sequenced isolates, respectively. At position 277, 59.0% of the sequences contained Lys, while 40.9% contained Arg. The variability at position 286 was lower, with Thr and Ala found in 69.1% and 29.0% of the sequences, respectively. Previous cross-sectional studies carried out with a large database containing HIV-1 subtype B *pol* sequences from patients treated in Spanish hospitals revealed an increased prevalence of Pro272, Arg277, and Thr286 in isolates from individuals failing therapy with abacavir/d4T and other nucleoside analogue combinations (20).

The p66 thumb subdomain plays a role in DNA polymerization by making important interactions with the minor groove of the template-primer through α -helices H (residues 255 to 268) and I (residues 278 to 286) (5, 16), while the p51 thumb subdomain contributes to the stabilization of the p66/p51 heterodimer (69). In addition, interactions between the p51 thumb subdomain and the RNase H domain of p66 appear to be essential for the conversion of the RT heterodimer from an inactive to an active form (45). Amino acid substitutions G262A and W266A within α -helix H are known to decrease RT processivity and frameshift fidelity (4) while impairing the RNase H-mediated removal of the polypurine tract during reverse transcription (51). These effects suggest that mutations in the RT thumb subdomain could alter the balance between excision and template RNA degradation in a way that could be similar to that reported for resistance-associated connection

subdomain and RNase H domain mutations (13, 18, 47, 48, 73). Here, we show the effects of thumb subdomain polymorphisms on viral replication capacity and provide insights into the mechanism by which Pro272, Arg277, and Thr286 confer a selective advantage for viral replication in the presence of excisable nucleotide analogues.

(A preliminary report of this work has been presented at the International HIV & Hepatitis Virus Drug Resistance Workshop & Curative Strategies, at Dubrovnik, Croatia, 8 to 12 June 2010, abstract 66 [5a].)

MATERIALS AND METHODS

Reverse transcriptases. In this study, the HIV-1_{BH10} RT has been arbitrarily designated the wild-type (WT) RT, and thumb subdomain polymorphisms have been considered mutations introduced in the sequence context of the BH10 enzyme (P272A, R277K, and T286A). Expression and purification of WT HIV-1_{BH10} RT, MAK_SSSY RT (HIV-1_{BH10} RT containing a Ser-Ser insertion between codons 69 and 70 and containing mutations M41L, A62V, T69S, K70R, and T215Y) and an RNase H-deficient RT (HIV-1 group O RT with mutations V75I/E478Q) were performed with modified versions of plasmid p66RTB, as previously described (1, 6, 10, 34, 36). Other mutant RTs were obtained by using the QuikChange site-directed mutagenesis kit (Stratagene), following the manufacturer's instructions. The double mutant M41L/T215Y was obtained with previously described mutagenic primers (10, 35). To introduce the P272A and R277K mutations, we used primers 5'-CAAGTCAGATTTACGCAGGGATTAAGTAAAGCAATTATGTAAAC-3' and 5'-GTTTACATAATTGCTTTACTTTAATCCCTGCGTAAATCTGACTTG-3'. To introduce the T286A mutation, the mutagenic primers were 5'-CTCCTTAGAGGACCAAGCACTAACAG A-3' and 5'-TCTGTTAGTGCTTTGGCTCTCTAAGGAG-3'. The introduced mutations were confirmed by DNA sequencing. RT p66 subunits carrying a His₆ tag at their C terminus were coexpressed with HIV-1 protease in *Escherichia coli* XL1 Blue to obtain p66/p51 heterodimers, which were later purified by ionic exchange followed by affinity chromatography (6, 34). RT concentrations were determined by active site titration as previously described (27).

Nucleotides and template-primers. Stock solutions (100 mM) of deoxynucleoside triphosphates (dNTPs), dideoxy-ATP (ddATP), and ATP were obtained from GE Healthcare. AZT triphosphate (AZTTP), and carboxy triphosphate (CBVTP) were purchased from Moravak Biochemicals (Brea, CA). Stavudine triphosphate (d4TTP) was obtained from Sierra Bioresearch (Tucson, AZ). Before use, nucleoside triphosphates were treated with inorganic pyrophosphatase (Roche) to remove traces of PP_i (33). DNA oligonucleotides 21P (5'-ATACTTTAACCATATGTATCC-3'), 25PGA (5'-TGGTAGGGCTATACATTCTGCAGG-3'), 31T (5'-TTTTTTTTTAGGATACATATGGTTAAAGTAT-3'), D38 (5'-GGGTCTTTTCTTACCTGCAAGAAATGTATAGCCCTACCA-3'), D38C (5'-GGGTCTTTTATCCCTGCAAGAAATGTATAGCCCTACCA-3'), D38T (5'-GGGTCTTTTCAATCCTGCAAGAAATGTATAGCCCTACCA-3'), and PR26 (5'-CCTGTCGGGCGCCACTGCTAGAGAT-3') and RNA oligonucleotides 31rna (5'-GGGUCCUUUCUACUGCAAGAAUGU AUAGC-3'), 31Trna (5'-UUUUUUUUUAGGAUACAUAUGGUUUAAAUG AU-3'), D38rna (5'-GGGUCCUUUCUACUGCAAGAAUGUUAUAGCC UACCA-3'), D38Crna (5'-GGGUCCUUUAUCCUGCAAGAAUGUAUA GCCCUACCA-3'), and T35rna (5'-AGAAUGGAAAUCUCUAGCAGUGG CGCCGAACAG-3') were obtained from Invitrogen. Oligonucleotides were labeled at their 5' termini with [γ -³²P]ATP (Perkin Elmer) and T4 polynucleotide kinase (Promega), and then annealed to their corresponding templates or primers depending on the experiment.

Chain terminator excision assays. RT-catalyzed DNA rescue reactions were performed with D38/25PGA, D38C/25PGA, and D38T/25PGA DNA duplexes, as previously described (32, 35). Briefly, the phosphorylated template-primer (75 nM) was preincubated at 37°C for 10 min in the presence of the corresponding RT at an active enzyme concentration of 60 nM in 50 mM HEPES buffer (pH 7.0) containing 15 mM NaCl, 15 mM magnesium acetate, 130 mM potassium acetate, 1 mM dithiothreitol, and 5% (wt/vol) polyethylene glycol 6000. Reactions were initiated by adding an equal amount of preincubation buffer containing the NRTI in its triphosphorylated form at a final concentration of 25 μ M. After the samples were incubated at 37°C for 30 min, rescue reactions were initiated by adding a mixture of all dNTPs in the presence of sodium PP_i (200 μ M) or ATP (3.2 mM) depending on the assay. In these assays, all dNTPs except dATP were supplied at a final concentration of 100 μ M. Since the next complementary dNTP (dATP in our assay conditions) has an inhibitory effect on the

rescue reaction, time course experiments of the unblocking and extension reactions were carried out with 1 μ M dATP. Rescue reactions were also performed with the RNA/DNA template-primers D38rna/25PGA and D38Crna/25PGA. These reactions were carried out under the same conditions as for the DNA duplexes, but the dNTP and inhibitor concentrations were 2-fold higher. Excision and extension reactions were carried out after preincubating the corresponding RT with template-primer and triphosphorylated NRTI for 10 min at 37°C. In experiments designed to assess the inhibitory effect of dATP, extension reaction mixtures were incubated for 15 to 40 min in the presence of different concentrations of dATP. In all cases, incubation times were within the linear range of the corresponding time course experiment. The reactions were stopped by adding an equal amount of sample loading buffer (10 mM EDTA in 90% formamide containing 3 mg/ml xylene cyanol FF and 3 mg/ml bromophenol blue). Products were resolved on a denaturing 20% (wt/vol) polyacrylamide–8 M urea gel, and primer rescue was quantified by phosphorimaging with a BAS 1500 scanner (Fuji) using the program Tina version 2.09 (Raytest Isotopenmessgeräte GmbH, Staubenhardt, Germany).

Pre-steady-state kinetics of the ATP-dependent excision reaction. For these experiments, primer 25PGA was blocked at its 3' end with d4TTP or CBVTP, using terminal deoxynucleotidyltransferase as previously described (33). Then, the free nucleotides were eliminated by repeated passage through a Quick Spin minicolumn (Roche), until the A_{260} remained unchanged. Blocked primers were labeled at their 5' terminus with [γ - 32 P]ATP and T4 polynucleotide kinase and annealed to template D38rna or D38Crna as described above, in order to obtain the template-primers D38rna/25PGA^{d4T} and D38Crna/25PGA^{CBV}. Single-turnover conditions were used to study the excision of d4T monophosphate (d4TMP) and CBVMP. Solutions containing 250 nM RT and 30 nM D38rna/25PGA^{d4T} or D38Crna/25PGA^{CBV} in RT buffer (15 mM HEPES [pH 7.0], 4 mM NaCl, 4 mM magnesium acetate, 130 mM KCH₃COO, 1 mM dithiothreitol, and 5% [wt/vol] polyethylene glycol) were mixed with equal volumes of RT buffer containing 6.4 mM ATP and 36 mM MgCl₂. Excision reactions were carried out for 0 to 60 min at 37°C. Aliquots were removed after defined incubation periods, quenched with sample loading buffer, and analyzed by denaturing polyacrylamide gel electrophoresis as described above. The formation of product (concentration of product [P]) over time was fitted to a single exponential decay: $[P] = A \times e^{-k_{\text{obs}} \times t}$, where k_{obs} is the apparent kinetic constant of the excision reaction. A linear regression was used to fit the data when the excision rates were low. In those cases, we assumed that the amount of active RT was identical to the template-primer concentration in the assay, since reactions were carried out with a large excess of RT relative to the RNA/DNA duplex.

Determination of dissociation equilibrium constants (K_d) for WT and mutant RTs and DNA/DNA and RNA/DNA template-primers. RTs were preincubated with increasing concentrations of the 5'- 32 P-labeled 25/38-mer DNA-DNA (2 to 60 nM) for 10 min at 37°C in 10 μ l of a buffer containing 100 mM HEPES (pH 7.0), 30 mM NaCl, 30 mM magnesium acetate, 130 mM KCH₃COO, 1 mM dithiothreitol, and 5% (wt/vol) polyethylene glycol. Reactions were initiated by the addition of 10 μ l of 1 mM dTTP to 10 μ l of the solution indicated above, and incubated at 37°C. In these experiments, the active RT concentration was around 3 nM. Aliquots of 4 μ l were then removed at 10, 20, 30, and 40 s, quenched with sample loading buffer, and analyzed by denaturing polyacrylamide gel electrophoresis as described above. The burst amplitudes (RT bound to template-primer at time zero) were plotted as a function of the template-primer concentration, and the data were fitted to a quadratic equation to obtain the equilibrium dissociation constant for RT binding to template-primer (37).

RNase H assays. RNase H activity was evaluated with 31Trna/21P and D38rna/25PGA complexes as previously described (1). Assays were carried out at 37°C in 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 5 mM MgCl₂, and 50 nM 32 P-labeled RNA/DNA template-primer, in the presence of 100 nM RT (active site concentration). Aliquots were removed at various times, quenched with equal volumes of gel loading dye, and analyzed by denaturing polyacrylamide gel electrophoresis as described above. In addition, the RNase H activity of WT and mutant RTs was determined with an AZT-terminated RNA/DNA substrate (i.e., T35rna/PR26) (8). Briefly, the 32 P-labeled T35rna/PR26 complex (80 nM) was blocked at the 3' end of the primer with AZTTP in the presence of a previously characterized RNase H-deficient RT (HIV-1 group O RT containing mutations V75I/E478Q [1]). Reaction mixtures were incubated for 60 min at 37°C in 50 mM Tris-HCl (pH 8.0), 50 mM KCl, 10 mM MgCl₂, and 60 μ M AZTTP in the presence of 30 nM RT. After heat inactivation of the enzyme, the free AZTTP was eliminated with a Quick Spin minicolumn (Roche) as described above. T35rna/PR26^{AZT} RNase H cleavage reactions were carried out at 37°C in 50 mM Tris-HCl (pH 8.0), 50 mM KCl, and 10 mM MgCl₂, containing 0.3 mM ATP, 20 nM 32 P-labeled RNA/DNA template-primer and the corresponding RT at a 200 nM active site concentration.

Recombinant virus and drug susceptibility tests. These recombinant virus and drug susceptibility assays were performed as described previously (32, 36). Briefly, full-length RT coding sequence DNA was amplified by PCR from the expression plasmids carrying the appropriate RT, using primers IN3 and IN5 (36). The PCR products were then cotransfected in MT-4 cells with an RT-deleted HXB2-D clone previously linearized with BstEII (28). Culture supernatants were harvested when the HIV-1 p24 antigen concentration surpassed 20 ng/ml. Progeny virus was propagated and titrated in MT-4 cells. The nucleotide sequence of the RT-coding region of the progeny virus was PCR amplified with the above-mentioned primers and checked for possible reversions or undesired mutations. The MT-4 cells and the deleted HXB2-D clone were obtained from the AIDS Reagent Program (Medical Research Council). RT inhibitors were obtained from the NIH AIDS Research and Reference Reagent Program. HIV-1 drug susceptibility data were obtained after infecting 30,000 MT-4 cells with 100 50% tissue culture infective doses (TCID₅₀) of virus at a multiplicity of infection of 0.003 by exposing the HIV-1-infected cultures to various concentrations of each drug (5-fold dilutions). After the MT-4 cells were allowed to proliferate for 5 days, the number of viable cells was quantified by a tetrazolium-based colorimetric method [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method] as described elsewhere (50).

Replication capacity assays. Viral replication kinetics of mutant viruses was assayed by infecting 5×10^6 peripheral blood mononuclear cells (PBMCs) (mixed from two healthy donors), previously stimulated with 3 μ g/ml of phytohemagglutinin (Sigma-Aldrich) and 10 U/ml of interleukin 2 with 500 TCID₅₀ of each viral stock (multiplicity of infection of 0.0001) (52). After incubation for 2 h at 37°C, cells were washed twice with phosphate-buffered saline and resuspended in RPMI 1640 medium supplemented with 20% fetal bovine serum and interleukin 2 (10 U/ml) at a final concentration of 1×10^6 cells/ml. Zidovudine (AZT) at different concentrations was added to the cultures when indicated. Viral replication was quantified by measuring HIV-1 p24^{Gag} antigen production in the culture supernatant for 10 days. Growth kinetics was analyzed by fitting a linear model to the log-transformed p24^{Gag} data during the exponential growth phase by maximum likelihood methods.

RESULTS

Thumb subdomain polymorphisms and their effects on viral replication. Our previous statistical analysis carried out with HIV-1 *pol* sequences obtained from patients treated in Spain showed that several RT thumb subdomain polymorphisms (i.e., Pro272, Arg277, and Thr286) were selected during therapy with nucleoside drug combinations such as d4T/abacavir, d4T/ddI, ddI/abacavir, d4T/lamivudine, and d4T/tenofovir (20). The largest levels of statistical significance were observed with the first two combinations. In addition, correlated pairs of mutations at positions 272 and 277 and positions 272 and 286 were observed in HIV sequences from patients failing therapy with d4T and ddI. Despite the statistical correlation, the effects of thumb mutations P272A/R277K/T286A on nucleoside (or nucleotide) RT inhibitor susceptibility were not significant in phenotypic assays (Table 1). All viruses tested were found to be susceptible to d4T, abacavir, ddI, tenofovir, lamivudine, and nevirapine. Statistically significant differences between the 50% inhibitory concentrations (IC₅₀s) obtained with WT and mutant HIV-1 were observed only in the case of AZT ($P < 0.01$ by Student's *t* test). Thus, recombinant HIV-1 containing RT mutations M41L/T215Y showed a decrease in susceptibility to AZT of about 3-fold. Interestingly, in the presence of TAMs (M41L/T215Y), the addition of the thumb subdomain mutations P272A, R277K, and T286A produced a slight decrease of the IC₅₀ for the inhibitor.

Viral replication kinetics assays carried out in PBMCs showed that mutations P272A/R277K/T286A had a negative effect on viral fitness (Fig. 1). Growth kinetics curves were analyzed by fitting a linear model to the log-transformed p24^{Gag} data during the exponential growth phase (see Fig. S1

TABLE 1. Susceptibility of HIV-1 constructs to RT inhibitors

RT	IC ₅₀ (nM) ^a						
	AZT	d4T	Abacavir	ddI	Tenofovir	Lamivudine	Nevirapine
WT	5.1 ± 2.6	315.6 ± 44.9	1,280.8 ± 242.4	2,210.4 ± 363.6	181.0 ± 38.6	1,014.1 ± 39.3	44.0 ± 15.7
P272A/R277K/T286A	6.1 ± 2.3 (1.2)	289.3 ± 50.9 (0.9)	1,169.8 ± 112.1 (0.9)	2,499.8 ± 894.9 (1.1)	221.1 ± 28.5 (1.2)	1,519.2 ± 486.0 (1.5)	23.1 ± 4.1 (0.5)
M41L/T215Y	14.5 ± 0.8 (2.8)	399.2 ± 54.3 (1.3)	1,526.1 ± 236.7 (1.2)	1,888.8 ± 604.0 (0.9)	264.4 ± 38.5 (1.5)	1,988.7 ± 628.1 (1.9)	22.8 ± 14.4 (0.5)
M41L/T215Y/P272A/R277K/T286A	10.7 ± 6.5 (2.1)	378.6 ± 90.3 (1.2)	1,768.2 ± 188.1 (1.4)	2,562.4 ± 710.3 (1.2)	261.4 ± 78.3 (1.4)	1,558.8 ± 854.2 (1.5)	29.1 ± 24.2 (0.7)

^a The IC₅₀ values shown are averages ± standard deviations of at least three tests, with each one performed six times. The fold increase in IC₅₀ relative to the wild-type HXB2 virus control carrying the RT sequence of BH10 is shown in parentheses.

in the supplemental material). The slope of p24 antigen production provides a reliable estimate of the viral replication capacity. As shown in Fig. 1, the addition of thumb subdomain mutations to HIV variants containing TAMs decreased viral replication capacity by 11 to 34%. These effects were more significant at higher concentrations of AZT. A similar trend was observed when mutations were introduced in a WT recombinant virus, although the negative effects were observed only in experiments carried out in the presence of 0.2 μM AZT. These results together with the observed associations between thumb subdomain polymorphisms and failure to therapies containing combinations of excisable NRTIs suggested a link between excision and the selection of HIV-1 variants containing Pro272/Arg277/Thr286.

Effects of mutations on the ATP-mediated excision of NRTIs from chain-terminated DNA/DNA and RNA/DNA template-primers. The ability of RTs to unblock NRTI-terminated prim-

ers was initially assessed with DNA/DNA template-primers (Fig. 2). An excision-proficient RT (i.e., MAK_SSSY [M41L/A62V/T69SSS/K70R/T215Y]) was used as a reference. In the presence of 3.2 mM ATP, MAK_SSSY was able to excise AZT monophosphate (AZTMP), d4TMP, CBVMP, and ddAMP, although ddAMP was removed at a very low rate. The ATP-dependent phosphorolytic activities of WT and mutant RTs (M41L/T215Y, M41L/T215Y/P272A/R277K/T286A, and P272A/R277K/T286A) were rather small in comparison with the activity shown by the MAK_SSSY RT. Although M41L/T215Y and M41L/T215Y/P272A/R277K/T286A RTs showed some activity with AZTMP-, d4TMP- and CBVMP-terminated primers, thumb subdomain mutations had a minor effect on excision. As previously observed with other RT variants, all enzymes tested showed similar excision efficiencies in the presence of PP_i (200 μM) (data not shown).

As in the case of DNA/DNA template-primers, the efficiency of the ATP-dependent phosphorolytic activity on RNA/DNA substrates in reactions catalyzed by MAK_SSSY RT followed the order AZTMP > d4TMP > CBVMP (Fig. 3). Control experiments carried out with d4T-terminated duplexes showed that the efficiencies of the unblocking and extension reactions catalyzed by all RTs were similar in the presence of 30 nM RT and 10 nM D38rna/25PGA (Fig. 3) to the efficiencies obtained in the presence of 24 nM RT and 30 nM D38rna/25PGA (data not shown). The ATP-mediated excision activity of M41L/T215Y and M41L/T215Y/P272A/R277K/T286A RTs was higher in reactions carried out with blocked RNA/DNA template-primers than with DNA/DNA substrates. Although both enzymes had remarkable excision activity, mutations P272A, R277K, and T286A produced a small reduction in the corresponding excision rates. These differences could not be attributed to their susceptibility to inhibition by the next complementary dNTP (see Table S1 in the supplemental material). Thus, in reactions catalyzed by M41L/T215Y and M41L/T215Y/P272A/R277K/T286A RTs, ATP-dependent rescue of primers terminated with d4TMP was inhibited by the next complementary dNTP at concentrations of around 4 μM. These values were similar to those reported for other RTs and obtained in reactions carried out with different DNA/DNA template-primers (10, 36, 42).

Interestingly, the introduction of P272A/R277K/T286A in an otherwise WT sequence context rendered an enzyme lacking ATP-dependent phosphorolytic activity in our assay conditions. These effects were observed in the presence of RNA/DNA duplexes containing primers terminated with AZTMP,

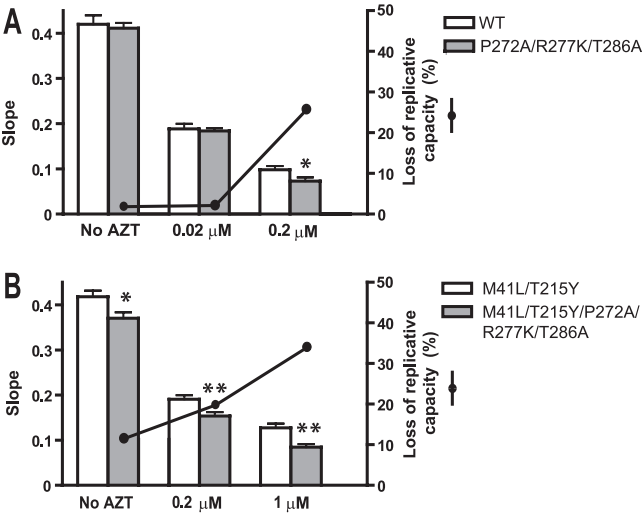


FIG. 1. Replication kinetics assay in the absence and presence of AZT. The slope of p24 antigen production of each virus after infection of PBMCs (mixed from two donors) is shown by the bars. Comparisons of WT versus mutant P272A/R277K/T286A virus and mutant M41L/T215Y virus versus M41L/T215Y/P272A/R277K/T286A virus are shown in panels A and B, respectively. The significance of the difference between slopes was calculated using the GraphPrism v. 4 software (*, $P < 0.05$; **, $P < 0.01$). Solid black circles represent the percentage loss of replication capacity of the recombinant HIV-1 variants containing the P272A, R277K, and T286A mutations in the absence (A) or presence (B) of M41L/T215Y mutations under different assay conditions.

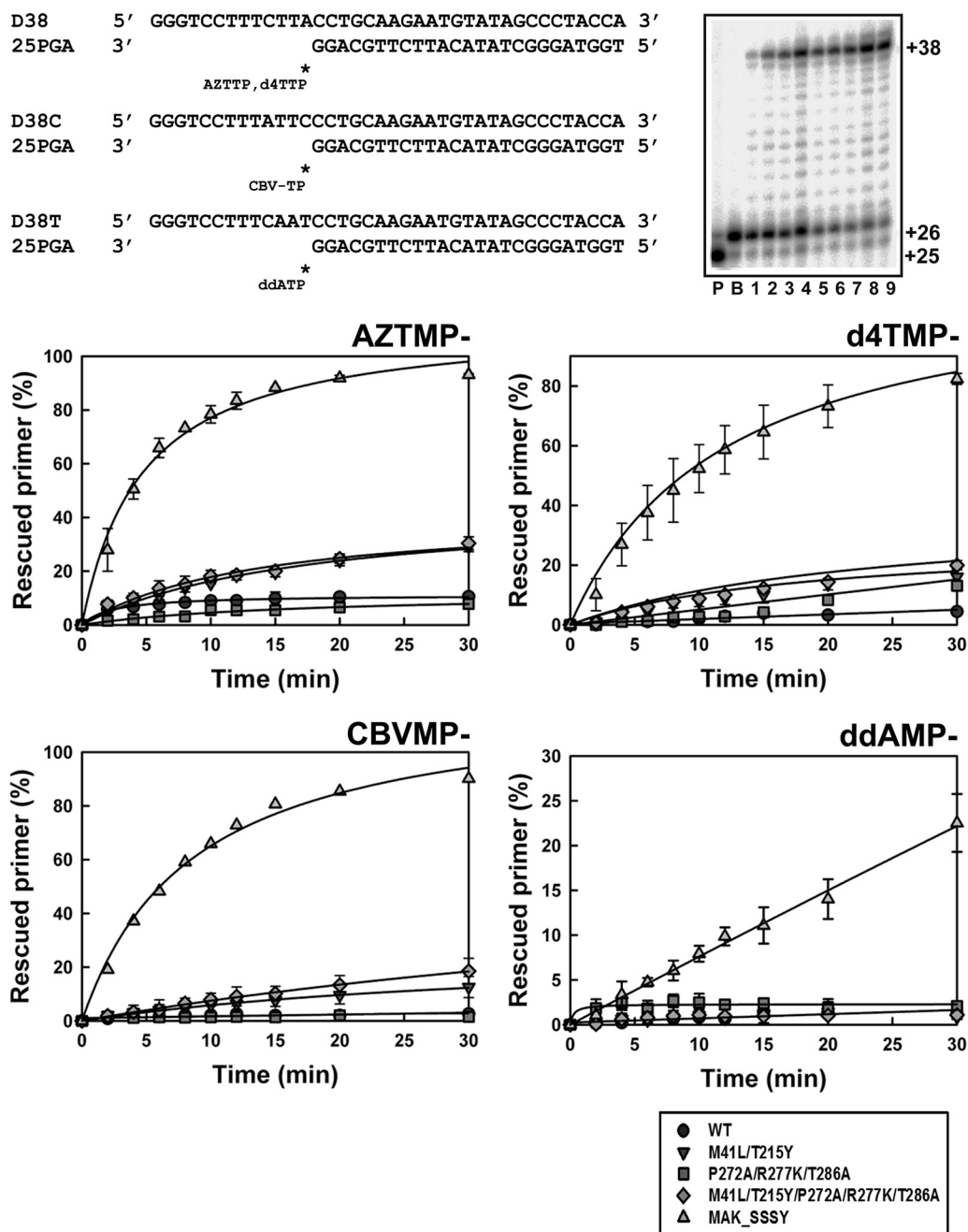


FIG. 2. ATP-mediated excision of AZTMP, d4TMP, CBVMP, and ddAMP from DNA/DNA template-primers by WT and mutant RTs. Reactions were carried out with 38/25-mer DNA/DNA heteropolymeric complexes (sequences shown at the top of the figure). First, the inhibitor was incorporated at position +1 (indicated with an asterisk) of the 25-nucleotide primer (lane P) to generate a 26-nucleotide product (lane B). Excision of the inhibitor and further primer extension in the presence of 3.2 mM ATP and a mixture of dNTPs lead to the formation of a fully extended 38-nucleotide product. A representative time course experiment of a primer rescue reaction is shown in lanes 1 to 9, which correspond to aliquots removed 2, 4, 6, 8, 10, 12, 15, 20, and 30 min after the addition of 3.2 mM ATP (gel in the top right corner of the figure). Graphs of time course experiments of primer rescue reactions initiated from inhibitor-terminated primers are given below. All dNTPs in the assays were supplied at 100 μ M, except for dATP whose concentration was 1 μ M. Template-primer and active RT concentrations in these assays were 30 and 24 nM, respectively. The values (averages \pm standard deviations [error bars]) were obtained from three independent experiments. The MAK_SSSY RT is an excision-proficient enzyme that contains a Ser-Ser insertion between codons 69 and 70 and contains mutations M41L, A62V, T69S, K70R, and T215Y.

d4TMP, or CBVMP. The presence of Pro272, Arg277, and Thr286 (as in the WT BH10 RT) conferred detectable excision activity, particularly on primers terminated with AZT and d4T, annealed to RNA templates (Fig. 3).

The d4TMP excision rates for WT and mutant P272A/R277K/T286A RT were determined under single-turnover conditions by using a d4TMP-terminated primer previously annealed to a 38-nucleotide RNA template in the presence of

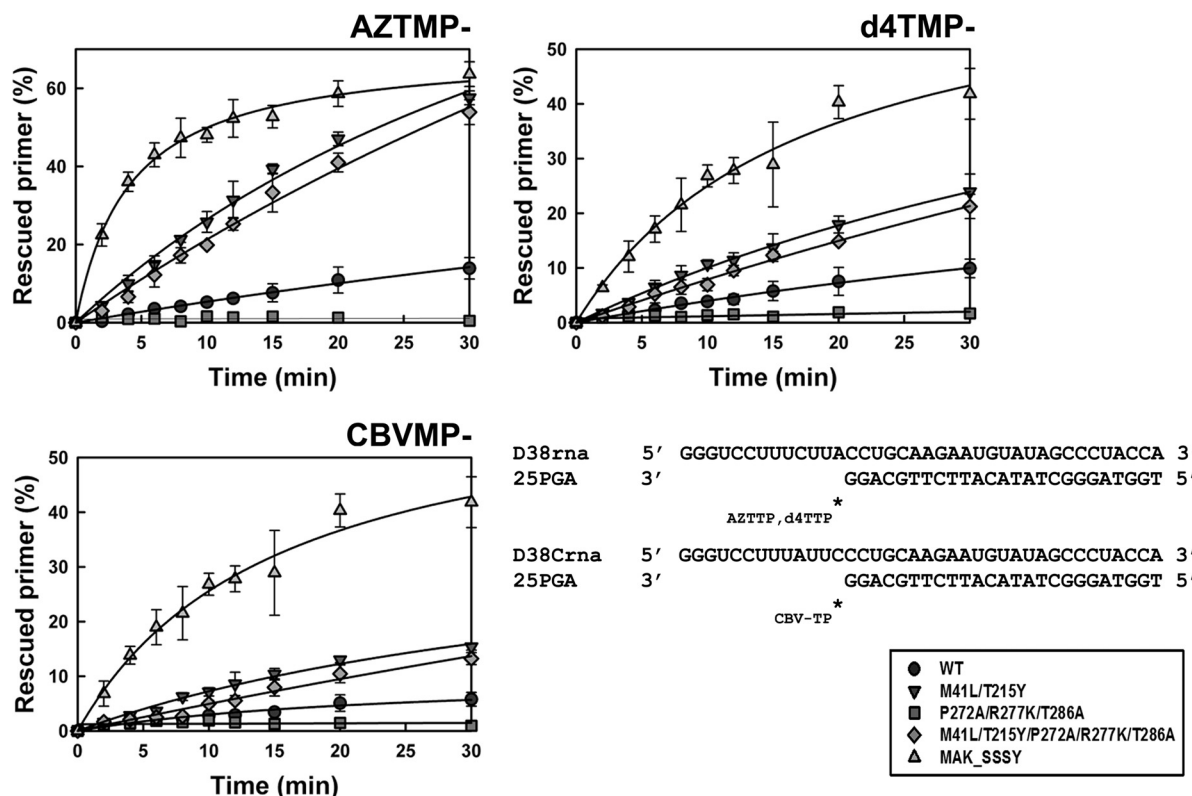


FIG. 3. ATP-mediated excision of AZTMP, d4TMP, and CBVMP from RNA/DNA template-primers by WT and mutant RTs. Time course experiments of excision reactions were carried out in the presence of 3.2 mM ATP. Template-primers used are indicated below. All dNTPs in the assays were supplied at 200 μ M, except for dATP whose concentration was 2 μ M. Template-primer and active RT concentrations in these assays were 10 and 30 nM, respectively. The values (averages \pm standard deviations [error bars]) were obtained from three independent experiments.

3.2 mM ATP but without dNTPs. The WT RT showed a d4TMP excision rate (k_{obs}) of 0.0032 min^{-1} , which was about 2.5 times higher than the value obtained for mutant P272A/R277K/T286A RT (Fig. 4). Both enzymes showed negligible activity on CBVMP-terminated RNA/DNA substrates. The differences between M41L/T215Y and M41L/T215Y/P272A/R277K/T286A RTs were not significant in these assays. Both enzymes showed remarkable d4TMP excision activity on RNA/DNA templates (k_{obs} around 0.08 min^{-1}), although they were less efficient in removing CBVMP (k_{obs} around 0.0036 min^{-1}).

RNase H activity of WT and mutant RTs. Thumb subdomain polymorphisms did not have a major impact on the RNase H activity as measured with the 31Trna/21P complex (Fig. 5A). Cleavage patterns monitored on the 5'-end-labeled RNA template revealed similar kinetics for reactions catalyzed by RTs with or without thumb subdomain mutations. No secondary cleavages were detected with the 31Trna/21P template-primer, under our assay conditions. Similar results were obtained with RNA/DNA template-primers used in rescue reactions (i.e., D38rna/25PGA) (Fig. 5A), although in this case the primary cleavage at position -18 occurred with higher efficiency. An increased frequency of secondary RNase H cleavages could diminish RNA/DNA duplex length and decrease the efficiency of NRTI excision. RNase H secondary cleavages occurring during the excision reaction were monitored with an AZT-terminated RNA/DNA complex in the presence of ATP. Catalytic rate constants for the cleavage of D38rna and 31Trna

were in the range of 0.97 to 1.67 min^{-1} and 0.49 to 0.71 min^{-1} , respectively (Fig. 5B). Differences between WT and P272A/R277K/T286A RTs and between M41L/T215Y and M41L/T215Y/P272A/R277K/T286A RTs were not significant in these assays ($P > 0.1$ by Student's t test). As shown in Fig. 5C and in Fig. S2 in the supplemental material, in an excision-competent mode, mutant RTs with P272A/R277K/T286A, M41L/T215Y, and M41L/T215Y/P272A/R277K/T286A had similar kinetics of formation of the -10 cleavage product. However, the WT enzyme showed 1.6-fold increased activity. Similar band patterns were observed for all tested RTs when the reactions were carried out in the absence of ATP (data not shown). As demonstrated for several RT connection and RNase H domain mutations (8, 48, 53), increased RNase H secondary cleavage efficiency correlates with lower levels of excision activity. Therefore, the higher ATP-mediated excision activity of WT RT relative to mutant P272A/R277K/T286A cannot be explained by invoking the effects of thumb subdomain mutations on polymerase-independent cleavages.

Effects of thumb subdomain polymorphisms on template-primer binding. The effects of thumb subdomain mutations on substrate binding were determined by measuring the equilibrium dissociation constants (K_d) for WT and mutant RTs and DNA/DNA and RNA/DNA template-primers. Both template-primers were substrates of the excision reactions shown in Fig. 2 and 3. However, the RNA/DNA template-primer used (i.e., 31Trna/25PGA) was a derivative of D38rna/25PGA lacking 7

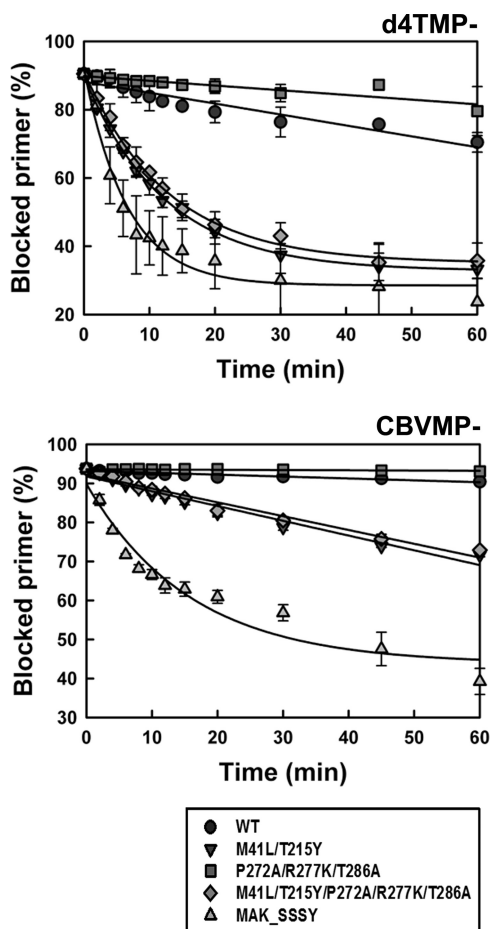


FIG. 4. Kinetics of the ATP-dependent excision of d4TMP and CBVMP from RNA/DNA template-primers. Time course experiments for the excision reaction of d4TMP- or CBVMP-terminated primers (26-mers) annealed to their corresponding 38-nucleotide RNA templates (30 nM) were determined in the presence of 3.2 mM ATP. The excision reaction was catalyzed by WT and mutant RTs (250 nM). The calculated k_{obs} values for the d4TMP excision reaction were $0.0032 \pm 0.0003 \text{ min}^{-1}$ for WT RT, $0.0014 \pm 0.0002 \text{ min}^{-1}$ for mutant P272A/R277K/T286A RT, $0.0810 \pm 0.0025 \text{ min}^{-1}$ for mutant M41L/T215Y RT, $0.0782 \pm 0.0044 \text{ min}^{-1}$ for mutant M41L/T215Y/P272A/R277K/T286A RT, and $0.153 \pm 0.018 \text{ min}^{-1}$ for the MAK_SSSY RT. For the excision of CBVMP, the k_{obs} values for mutants M41L/T215Y, M41L/T215Y/P272A/R277K/T286A, and MAK_SSSY RTs were $0.0038 \pm 0.0003 \text{ min}^{-1}$, $0.0035 \pm 0.0002 \text{ min}^{-1}$, and $0.0629 \pm 0.0138 \text{ min}^{-1}$, respectively.

nucleotides at the 3' end of the RNA. These nucleotides were eliminated, since the corresponding RNase H primary cleavage (at position +18) occurs early during the ATP-mediated excision reaction (Fig. 5). As shown in Table 2, thumb subdomain polymorphisms had a minor impact on the K_d for DNA/DNA complexes (38/25-mer), with values around 2 to 3 nM. However, WT and mutant M41L/T215Y showed about 2-fold-higher affinity for RNA/DNA than their homologous counterparts having the thumb subdomain mutations P272A, R277K, and T286A. Primer extension assays carried out with 31rna/25PGA were consistent with the K_d measurements (Fig. 6). At low concentrations of template-primer (i.e., 1 nM), the amount of fully extended product was about 2-fold higher for mutant

M41L/T215Y RT than for M41L/T215Y/P272A/R277K/T286A RT, while at higher concentrations (i.e., 10 nM), differences in the efficiency of the primer extension reaction were very small. On the other hand, thumb subdomain mutations had a relatively minor influence on RNase H cleavage patterns and kinetics in reactions carried out in the presence of low concentrations of RNA/DNA duplexes (i.e., 1.5 nM D38rna/25PGA) (see Fig. S3 in the supplemental material). Interestingly, the results of the primer extension assays were in agreement with fitness assays showing that in the absence of antiretroviral drugs, HIV clones containing the M41L/T215Y RT had a higher replication capacity than their homologous HIV-1 variants containing RT mutations M41L/T215Y/P272A/R277K/T286A (Fig. 1).

DISCUSSION

The HIV-1 RT thumb subdomain residues 255 to 286 form a characteristic helix-turn-helix secondary structure (termed the helix clamp) that exhibits sequence homology with other nucleic acid polymerases (22). In p66, the two α -helices (H and I) interact with the template-primer, while in p51 they play a structural role by contributing to the stabilization of the RT heterodimer (23, 29, 40, 69). Our previous statistical analysis carried out with HIV-1 *pol* sequences obtained from patients treated in Spain showed that thumb subdomain polymorphisms were selected during therapy with NRTIs (20). In most cases, polymorphisms were associated with TAMs and the most significant associations were detected with samples from patients failing therapy with combinations of excisable NRTIs (e.g., abacavir/d4T, d4T/ddI, etc.). In those studies, the frequencies of Pro272, Arg277, and Thr286 in the naïve population were 45.0%, 25.7%, and 54.5%, respectively. However, those numbers increase up to 82.6%, 63.0%, and 91.3%, respectively, among isolates from patients failing therapy with abacavir/d4T (20).

Phenotypic assays carried out with mutant RTs bearing thumb subdomain mutations like P272A, R277K, and T286A failed to detect differences in their susceptibility to NRTIs. This result was not unexpected, assuming their accessory role in resistance and the fact that assays were carried out in MT-4 cells. These cells contain high concentrations of dNTP that are known to counteract nucleotide excision mechanisms of resistance, particularly for abacavir, d4T, ddI, and tenofovir (64, 67). However, in phenotypic assays, mutant HIV-1 carrying the M41L/T215Y/P272A/R277K/T286A RT was found to be slightly more susceptible to AZT than its homologous counterpart containing the double mutant M41L/T215Y polymerase. Moreover, viral replication capacity assays carried out with PBMCs underlined these differences and showed that HIV-1 clones bearing Pro272, Arg277, and Thr286 in their RT-coding region had an increased growth rate in the presence of AZT. A phylogenetic tree obtained with the HIV-1 *pol* sequences derived from our previously reported cross-sectional study (20) showed the clustering of isolates obtained from patients failing therapy with abacavir and d4T bearing Pro272, Arg277, and Thr286 in their RT-coding region (Fig. 7). Despite the lack of sequence information from the connection and RNase H domains of the RT, these data argue in favor of the selection of

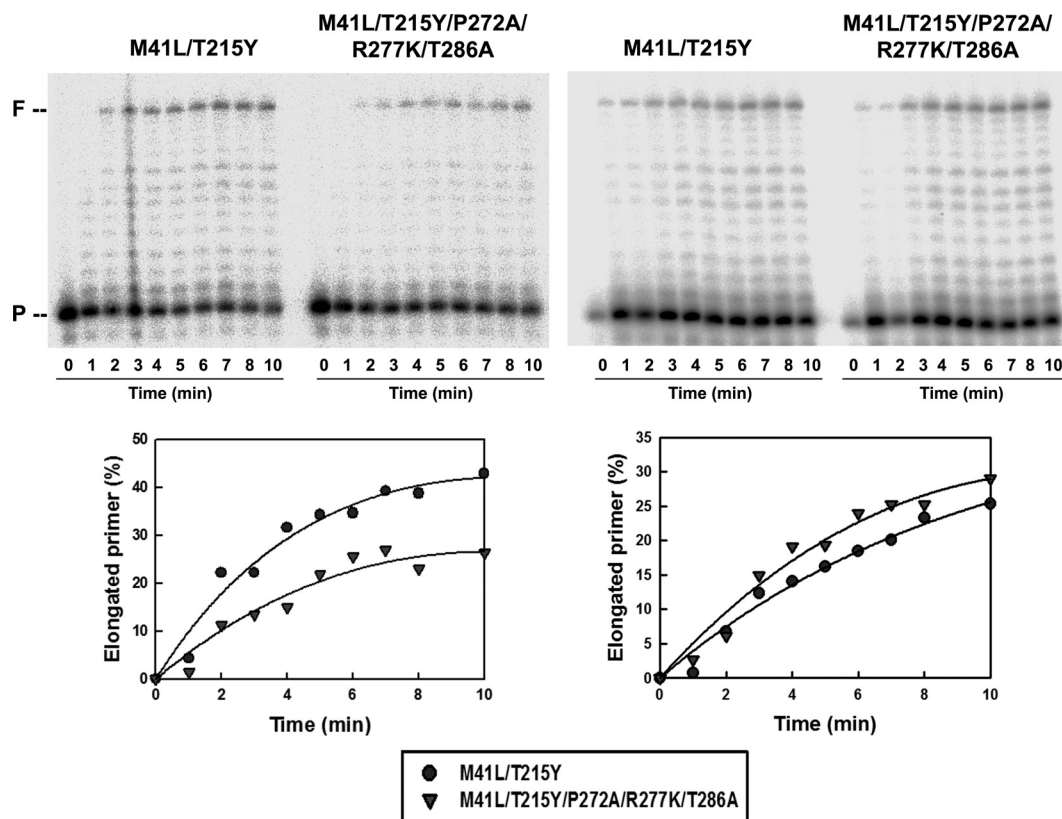


FIG. 6. Extension of an unblocked DNA primer 25PGA by mutant RTs M41L/T215Y and M41L/T215Y/P272A/R277K/T286A in the presence of an RNA template (31rna). Reactions were carried out with template-primer concentrations of 1 nM (left panel) and 10 nM (right panel) in 50 mM HEPES buffer (pH 7.0) containing 15 mM NaCl, 15 mM magnesium acetate, 130 mM potassium acetate, 1 mM dithiothreitol, 5% (wt/vol) polyethylene glycol 6000, and 200 μ M each dNTP except dATP, which was supplied at 2 μ M. The RT concentration used in these assays was 3 nM (active site concentration). P, primer; F, full-length product.

secondary cleavage kinetics. These effects were demonstrated in the presence of TAMs. We tested the effects of thumb subdomain polymorphisms on the kinetics of accumulation of RNase H cleavage fragments, using three different RNA/DNA complexes and different assay conditions. However, none of the experiments revealed significant differences between RTs containing or lacking thumb subdomain mutations. The G333D mutation in the connection subdomain has also been found to promote resistance to AZT in the presence of TAMs without affecting RNase H activity (74), but unlike in the case of the thumb mutations, an increased excision was reported for both RNA/DNA and DNA/DNA substrates.

In agreement with the results of rescue assays, we found that the apparent K_d for DNA/DNA was not influenced by the presence of thumb polymorphisms. However, in the case of RNA/DNA substrates, WT and M41L/T215Y RTs showed higher affinity for the template-primer than their homologous enzymes having thumb subdomain mutations. These differences could explain the lower efficiency of rescue reactions catalyzed by enzymes containing mutations P272A/R277K/T286A. It should be noted that when the amino acid substitutions M41L and T215Y were present, the negative effect of P272A/R277K/T286A on the efficiency of the rescue reaction was barely detectable at template-primer concentrations around 10 nM but become more significant at lower RNA/

DNA concentrations (i.e., at 1 nM, as shown in Fig. 6). A reduction in template-primer affinity could influence nucleotide excision by decreasing the availability of NRTI in an excision-competent conformation but could also decrease the efficiency of the primer extension reaction after removal of the chain-terminating inhibitor. In the absence of M41L and T215Y, thumb subdomain polymorphisms could also affect the orientation of the blocked primer, thereby influencing excision by altering the geometry required for the attack of the PP_i donor on the terminal phosphodiester bond.

Our results raise the question of how thumb subdomain mutations could affect NRTI excision. The analysis of the crystal structure of HIV-1 RT complexed with an RNA/DNA substrate (60) revealed that Arg277 and Thr286 of the p66 subunit are close to the RNA template strand (Fig. 8). The distances between an oxygen substituent of the phosphate group linking template nucleotides -7 and -8 and the amino groups of Arg277 are around 3.3 Å, while the side chain of Thr286 points toward the RNA template. A distance of 4.4 Å is observed between the hydroxyl group of Thr286 and the 2'OH of the ribose at position -8 of the template. On the other hand, Pro272 is away from the template-primer and could play a role in facilitating the appropriate folding of the thumb subdomain. However, single amino acid substitutions at this position were shown to affect template-primer binding and RT processivity

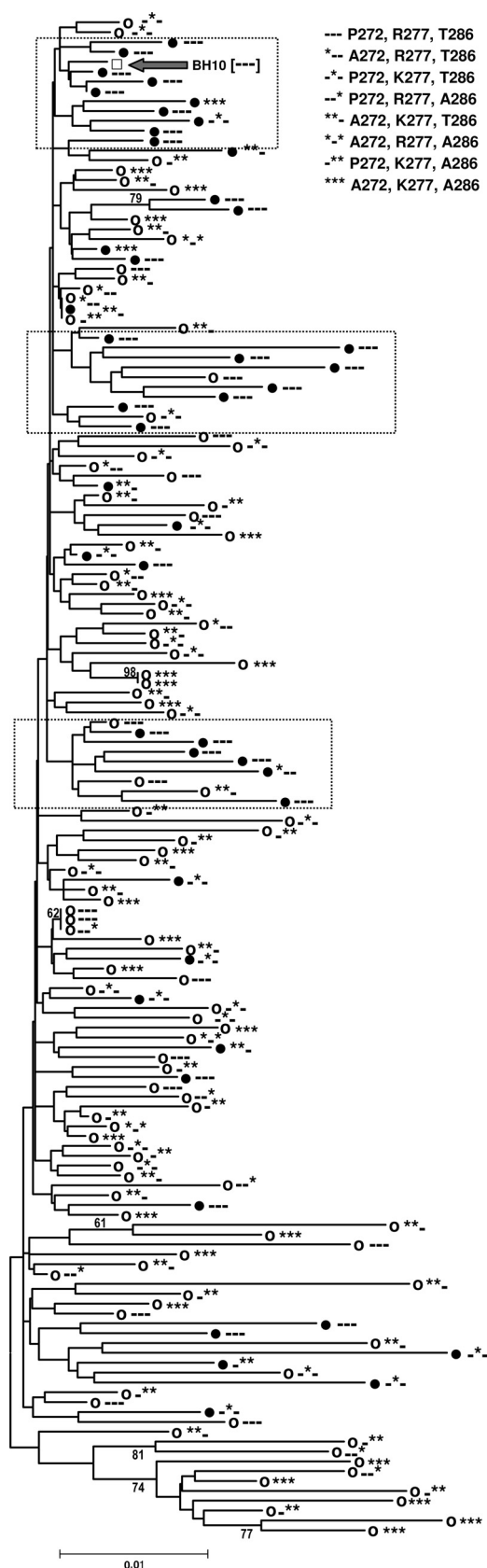


FIG. 7. Evolutionary relationships of HIV-1 protease and RT-coding sequences of isolates from naïve patients and individuals failing

(70). In p51, the relevant thumb subdomain polymorphisms do not appear to be involved in interactions with the template-primer, although structural information is still limited. Thr286 could play a role in stabilizing the RT heterodimer (40).

Compared with the HIV-1 RT structure containing RNA/DNA, the analysis of crystal structures containing DNA/DNA duplexes (16, 23) revealed that there were less extensive contacts between the RT template grip residues (i.e., α -helix I [residues 278 to 286]) and the DNA/DNA template-primer. Furthermore, in these two structures, the side chain of Arg277 points away from the DNA/DNA substrate, although Thr286 appears in a similar position as in the structure containing RNA/DNA, but closer to the sugar-phosphate backbone at positions -8 and -9 of the template (interatomic distances of 3.7 to 5.4 Å). Similar conformations were observed in the structures of HIV-1 RT with pre- and posttranslocation AZTMP-terminated DNA/DNA (58). Taken together, structural data are consistent with our findings regarding the loss of affinity for RNA/DNA complexes of mutant RTs containing R277K and other amino acid substitutions in the polymerase thumb subdomain. Interestingly, another mutation in α -helix I of the thumb subdomain (i.e., R284K) has been found to be associated with the accumulation of TAMs in treated patients (9, 71). Arg284 lies close to the RNA template, and as in the case of the thumb polymorphisms described in our study, could affect NRTI excision by altering interactions with the template-primer. Despite the compelling evidence in favor of excision as the molecular mechanism facilitating the selection of thumb polymorphisms under treatment with NRTIs, we cannot exclude the potential contribution of polymorphisms to RT heterodimer stability *in vivo*, either by changing interactions between p66 and p51 (40, 45, 69) or by altering the susceptibility of RT subunits to cleavage by the viral protease (17, 49).

In summary, our studies provide strong evidence for a mechanism that involves RNase H-independent contributions to increases in the efficiency of NRTI excision, and in turn, a selective advantage to these drugs; as well as for the effects of thumb subdomain polymorphisms Pro272, Arg277, and Thr286 on RNA/DNA template-primer affinity that may result in a selective advantage both in the presence or in the absence of RT inhibitors. Although the contribution of thumb subdomain

therapy with abacavir and d4T. Sequences (GenBank accession numbers HM460345 to HM460497) from isolates obtained from patients failing treatment with abacavir and d4T and from untreated patients (20) are represented by solid black circles and open circles, respectively. The WT BH10 sequence, encoding Pro272, Arg277 and Thr286 in the viral RT, is represented by the open square. Clusters of isolates from patients failing therapy with abacavir/d4T are boxed. The evolutionary history was inferred by the neighbor-joining method (57) by using p-distances in the substitution model. The bootstrap consensus tree was inferred from 2,000 replicates (19). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (2,000 replicates) are shown next to the branches (19). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. A total of 778 positions were considered in the final data set (all positions containing gaps and missing data were eliminated prior to analysis). Phylogenetic trees were obtained by using MEGA4 software (66).

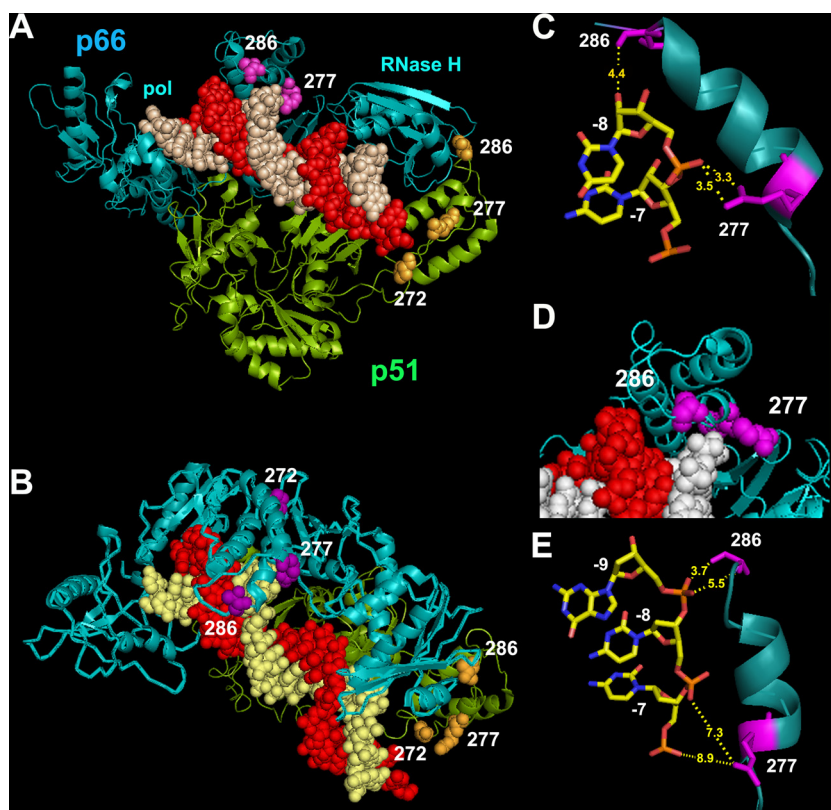


FIG. 8. Structure of HIV-1 RT in complex with RNA/DNA. The RT subunits are represented as ribbon diagrams (cyan for p66 and green for p51). The DNA primer is shown in red, while the RNA template is shown in white (in panel A) or yellow (in panel B). The side chains of relevant residues of the thumb subdomains of p66 and p51 are shown in magenta and orange, respectively, using a Corey-Pauling-Koltun (CPK) model. Structures were drawn with the PyMOL molecular viewer (<http://www.pymol.org>). Atom coordinates were taken from PDB file 1HYS (60). Side and top views are shown in panels A and B, respectively. (C) Relevant distances between the side chains of Arg277 and Thr286 and the RNA template. (D) Locations of positions 277 and 286 in the p66 subunit of a binary complex of HIV-1 RT and a DNA/DNA duplex (PDB file 2HMI) (16). The template is shown in white, and the primer is shown in red. (E) Relevant interatomic distances between the side chains of Arg277 and Thr286 and the template DNA backbone.

polymorphisms to drug resistance may not be significant, their effects on viral fitness can be relevant for the selection of NRTI-resistant HIV variants. In this context, accessory mutations in the connection and RNase H domains of the RT could also modulate the effects of thumb subdomain polymorphisms, arguing in favor of the inclusion of C-terminal portions of RT in clinical genotypic and phenotypic assays. Further studies on the role of accessory mutations should be helpful for designing prediction algorithms for the viral replication capacity.

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